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Validation of the use of doubly labeled water for measuring metabolic rate in Timber Rattlesnakes (*Crotalus horridus*)

An Honors Thesis submitted in partial fulfillment of the requirements of Honors Studies in Biology

By

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Contents

Abstract

The doubly labeled water method is an isotopic technique for measuring field metabolic rate and water flux rates of free-living animals. We present a validation of the use doubly labeled water for measuring metabolic rate and water loss in Timber Rattlesnakes (*Crotalus horridus*). For this study seven animals of varying body size were used with masses ranging from 148 to 650 grams. Prior to dosing, blood samples were taken to establish background isotope levels for each animal. Snakes were injected with water enriched with isotopes of oxygen (^{18}O) and hydrogen (^{2}H, deuterium). The injected isotopes were then allowed to equilibrate with the animals' body water pool for 3 hours and then a second blood sample was taken. Following the second blood sample, animals were placed into metabolic chambers and metabolic rates (VCO_2 and VO_2) were measured using open-flow respirometry for 20 days. During metabolic measurements water loss for each snake was estimated via Drierite uptake. On the 20^{th} day, final blood samples were taken to determine isotope turnover rates. Measured CO_2 production by gas exchange will be compared to CO_2 production calculated from isotopic data. Water flux measured by Drierite uptake will be compared to water flux calculated from isotopic data. Despite years of application of the DLW technique, validation studies are few: our data are the first such validation in pitvipers.

Introduction

Everything an organism does, both physiologically and behaviorally, depends on energy expenditure. As such, the evolution of organisms is often constrained within the range of what is energetically feasible (Butler et al. 2004). Specifically, there is a dependent relationship between energy utilization and the genetic fitness of organisms: all other things being equal, individuals that obtain and process energy with the greatest efficiency, as well as balance it against other factors encroaching on their reproduction and survival, will have the greatest genetic fitness (Tolkamp et al. 2002). The measurement of an organism's metabolic rate is therefore essential to understanding its physiological, behavioral and evolutionary features.

In general, energetics studies the flow and transformation of energy. The transfer of energy from storage to allocation can be measured in the form of heat loss, which is known as direct calorimetry. Direct calorimetry is a direct measure of heat production through means of enclosing an animal within a small chamber and determining the amount of heat produced. However, accurately and directly measuring the heat production of small organisms is very difficult (Speakman 2014). The low metabolism and subsequent low heat production renders ectotherms unfavorable subjects for this method. Indirect calorimetry is based on the fundamentals of oxidation reactions; where the products and reactants of aerobic metabolism are measured. Operating in accordance to the theory of mass conservation, this method utilizes the concept of mass balance in terms of aerobic metabolism: O_2 + fuel = CO_2 + H_2O. Often indirect calorimetry is preferred because it is relatively inexpensive and easy to determine an organism's exact oxygen consumption and/or subsequent carbon dioxide production (Lighton 2008).

The doubly labeled water (DLW) method is a type of field-based respirometry that utilizes stable isotopes to measure field metabolic rates (CO_2 production), water fluxes, and

feeding rates of free-living animals as well as in studies concerning metabolic rates for specific activities (Lifson and McClintock, 1966; Nagy, 1989; Speakman, 1997). These features may then be used to calculate the daily energy expenditure and time–energy budgets of an organism, which then provides insight into its daily food requirements and allocation of energy to various activities (i.e. growth, reproduction, foraging). Since the initial development of the DLW method in the 1950s and 1960s, it has become a widely used method in a variety of taxa (Speakman, 1997; Nagy et al., 1999; Nagy, 2005); however, it has been rarely used in pitvipers (Beaupre 2008).

In contrast to radioactive isotopes, stable isotopes are isotopes that do not decay into other elements and pose no known physiological risks (Fry 2006). The DLW method requires injecting an animal with water enriched with stable isotopes of oxygen (^{18}O) and hydrogen (^{2}H, deuterium, or ^{3}H, tritium) (Jones et al. 2009). Prior to the initial injection, a blood sample is taken to establish a baseline (of the isotopes naturally occurring in the animal). The injected isotopes are then allowed to equilibrate with the animal's body water, and then a second blood sample is taken. Following, the animal is allowed to carry out its day-to-day activities, but will later be recollected for a final blood sample. Differences between the initial and final isotope levels will yield an estimate for field metabolic rate (FMR) and water loss (given the animal's respiratory quotient or measured respiratory exchange ratio is known) (Jones et al. 2009). The effectiveness of the DLW method can be determined by simultaneously measuring metabolic rate using DLW and respirometry (Porter at al. 2004).

Timber Rattlesnakes (*Crotalus horridus*) are great indicators of longer-term trends due to their ability to survive in low-energy environments (Beaupre 2008). Specifically, they are able to endure prolonged periods of inactivation; therefore, this relative stability allows them to be

particularly suited for using DLW to study their energetics. Their relatively low metabolic rates allow for moderate water turnover rates, which prevent the rapid washout of isotopes (Jones et al. 2009). The ability to conserve water and withstand long periods of dehydration also allows this species to withstand the conditions of the study and permits the duration of the testing period to extend out to the isotopes' estimated half-life (Beaupre 2008). All of these characteristics exhibit the efficiency and validity of studies involving the analysis of environmental effects on snake species. Using them, this study will validate the use of the doubly labeled water method in measuring the field metabolic rate of Timber Rattlesnakes.

Methods

For this study seven Timber Rattlesnakes (*Crotalus horridus*) of varying body size were used with masses ranging from 148 to 650 grams. The general DLW technique used in this study closely followed Nagy (1983) with the exception of using deuterium (^{2}H) instead of tritium (^{3}H).

The snakes were fasted for 14 days before blood samples were taken to establish background isotope levels for each animal. All blood samples (each approximately 0.5 mL) were taken from the caudal vein (Bush and Smeller, 1978) and immediately added to 2 mL screw-cap watertight microcentrifuge tubes containing dehydrated EDTA. Samples were then spun down in a high-speed centrifuge for 30 minutes to separate the whole blood into its component parts. Plasma was removed and added to a labeled MicroLiter tube, sealed with Parafilm, and frozen until analysis. Snakes were then injected intraperitoneally with water enriched with isotopes of oxygen (^{18}O) and hydrogen (^{2}H, deuterium). The estimated dosage was calculated as follows:

mass of animal (kg) * .70 [assuming total body water = 70% body mass] = body water (kg)

body water (kg) * .561 (g/kg) [target dosage concentration] = estimated dosage (g)

Actual mass of the injectate was measured and recorded, as well as the time of the injection. The injected isotopes were allowed to equilibrate with the animals' body water pool for 3 hours and then a second blood sample was taken.

A dilution validation was also performed to ensure that the isotope equilibrated properly. A background sample of tap water was taken. Three additional samples of known water mass with a range of masses from 117 to 228 grams were collected and each was injected with the appropriate estimated dosage. The actual mass of the injectate was then measured and recorded,

as well as the time of the injection. The background tap water and diluted samples were transferred to MicroLiter vials and frozen until analysis.

Following the second blood sample, each animal was placed inside a metabolic chamber. The size of each chamber (500 mL – 2300 mL) was determined by the size of each animal, in an effort to minimize the amount of dead space in each chamber. All metabolic chambers were kept inside an environmental chamber to ensure a stable environmental temperature (25˚C). A modified version of Beaupre and Zaidan's (2001) open-flow respirometry setup was then used to measure metabolic rates (V_{CO_2} and V_{O_2}); see Figure 1. Air scrubbed of CO_2 and water flowed from a purge gas generator through a regulator before entering a manifold and being diverted into each chamber. The flow rates of air through each animal's chamber were regulated to 450 mL/min. Each chamber was connected to a channel line that linked to its own corresponding Drierite column where ex-current air was scrubbed of water vapor before entering a multiplexer. Air leaving the multiplexer was pushed through an O_2 analyzer (Sable Systems), which measured O_2 as ppm. After the O_2 analyzer, air then entered a mixing chamber along with scrubbed air from the Mass Flow Controller (MFC) (Sable Systems) at 675 mL/min to dilute the incoming gas stream to a total flow rate of 1125 mL/min before being subsampled. With a subsampler (Sable Systems) set to 450 mL/min, the gas stream was pulled though a second Drierite column and then a CO_2 analyzer (LI-COR) that measured CO_2 as ppm. Each chamber was measured for 7.5 minutes every hour over the course of 20 days (473 hours). A sample was taken every 5 seconds, accumulating to a total of 90 data points every hour per chamber. Switching between chambers was dictated by a computer-controlled multiplexer. Along with each animal chamber, a baseline (line without a chamber or Drierite column) was measured at the top and bottom of each hour for 3.75 min each. The computer program, Expedata (Sable Systems) was used to control

the multiplexer, record data, and process data for analysis. In total the program recorded the following every 5 seconds per chamber: subsampler A flow rate (mL/min), O_2 (ppm), subsampler B flow rate (mL/min), CO_2 (ppm), and the temperature of the chamber (˚C).

To determine water loss per animal, each drying column was filled with approximately 30 mL of recharged indicating Drierite and the initial mass of each was measured. Final Drierite column masses were then determined post-run. The pre- and post-run difference for each chamber was then summed together to get an approximate mass for total water loss for each animal. Drierite columns were examined at least every 12 hours and changed in accordance to observed turnover; columns were changed a minimum of once during each 24-hour period. Spent Drierite was recharged in an oven heated to 210-230˚C for one hour.

On the 20^{th} day all animals were disconnected from the respirometer, weighed, and final blood samples were taken. Measurements of ^{2}H and ^{18}O enrichment of all blood and water samples were performed at the University of Arkansas Stable Isotope Laboratory (UASIL). In summary, each sample was injected into a high-temperature combustion chamber and subsequently reduced to CO and H_2 gas. An isotope-ratio mass spectrometer then analyzed these gases to measure Delta ^{2}H and Delta ^{18}O. These values were then adjusted and used in the equations of Nagy (1980) and Nagy and Costa (1980) to calculate field metabolic rate and body water flux. Measured CO_2 production from open-flow respirometry was compared to the CO_2 production calculated from the isotopic data. During metabolic measurements, water loss for each snake was estimated via Drierite uptake, which in combination with water flux measured by mass loss was compared to water flux calculated from the isotopic data.

A total of 473 hours of open-flow respirometry data was acquired from each animal. Before analysis, raw data was prepared with Expedata. Baseline O_2 and CO_2 values collected at

the top and bottom (3.5 min each) of each hour provided a control for each chamber per hour. Baseline corrections were then made by comparing each gas reading sample to the baseline.

The open-flow respirometry setup (see Figure 1) positioned the CO_2 meter downstream of O_2 meter, which resulted in an approximately 1 second lag between measurements. If left unattended the lag shift would cause a misalignment of the O_2 and CO_2 peaks. To ensure the respiratory quotient (RQ) was calculated correctly ($RQ = V_{CO_2}/ V_{O_2}$), CO_2 measurements were shifted to left by 1 second.

A sampling rate of one value per five seconds resulted in a total of 90 data points every hour per chamber. However, each time there was a switch between chambers there was a corresponding dip in the flow rate and also a small lag in the system. To account for this, a specialized Macro written for Expedata cut each hour's sampling for each chamber to the last 70 sets of data of the total 90 samples taken. Thereby the last 350 seconds (5.833 min) of each chamber's data was used for calculations per hour.

In addition to trimming the data set, the Macro also processed the raw data and the subsequent values were generated: Frac CO_2, Frac O_2, V_{CO_2} (mL/h) V_{O_2} (mL/h), CO_2 area (mL), O_2 area (mL), and RQ. Gas concentrations were recorded in ppm but then converted to fractional concentration for ease of calculations. To calculate total CO_2 production (mL), V_{CO_2} (mL/h) was first determined using the following equation:

$$\dot{V}_{CO_2} = (f_e - f_i) \times FR \times 60$$

where V_{CO_2} is in milliliters per hour, f_e is the fractional concentration of CO_2 in the chamber ex-current air, f_i is the fractional concentration of CO_2 in the chamber in-current air, FR is the flow rate in milliliters per minute, and the factor 60 converts (mL/min) to hourly rates (mL/h).

The total CO_2 production (mL) for each animal was then determined as the sum of the

integral V_{CO_2} (mL/h) for every sample collected from each animal over the 20 day period. The Macro took an hourly average for each piece of data recorded or generated; with exception of total CO_2 and O_2 (mL), which were summed for each hour.

To validate the observed V_{CO_2} (mL/h) as within theoretical limits and to validate the calculation of total CO_2 production (mL), a predictive V_{CO_2} value for each animal was calculated for comparison as a function of its body mass and the temperature of the chamber using the following equation:

$$\dot{V}_{CO_2} = 0.00124 W^{.777} 10^{.0590T}$$

such that W is mass in grams and T is temperature in degrees Celsius (Beaupre and Zaidan 2001). Each average was accompanied by a 95% confidence interval (CI). The metabolic difference was then determined by calculating the difference between the observed and predicted V_{CO_2} values.

Results

As documented in Table 1, the average water loss for all seven subjects was 35.48±16.24 grams (mean±1 SD). Every subject fell within one standard deviation except CRHO-06, who fell within one and two standard deviations from mean water loss.

The results from the respirometry analyses are summarized in Tables 2 and 3. Table 2 provides a comparison between the average observed V_{CO_2} (3.993±1.672 mL/h) and the average predicted V_{CO_2} (4.399±1.586 mL/h). The metabolic difference (observed – predicted V_{CO_2}) for each animal as well as the two averages (-0.406±0.667 mL/h) was also included. The average mass loss experienced by each animal was 37.91±18.31 grams, and the average Drierite water loss for each was 35.48±16.24 grams. The average mass loss difference (mass loss – water loss) was determined to be 2.43±2.65 grams. Summed V_{CO_2} area values yielded an average total CO_2 (mL) production of 1860.03±779.58 mL.

Results from the stable isotope lab in Table 4 indicate an analytical error. The isotope data accurately depicts isotope enrichment between the background samples and the post-injectate equilibration samples (BKG vs. S1 samples); however, subsequent isotope levels for the final blood samples (S2) are irrational. For instance, two samples show increased $\delta^{18}O$ isotope levels between the post-injectate equilibration (S1) and final blood samples (S2). Several samples also indicate a very minimal deflection in isotope levels over the 20 day trial period. Isotope data was expected to have yielded a significant deflection in isotope levels between the post-injectate equilibration sample (S1) and the final sample (S2) in correspondence to measured CO_2 production by gas exchange and water flux measured by Drierite uptake. The exact causes of these biologically irrational values are being investigated; once resolved the values will be used as intended.

Discussion

There was an observed positive correlation between column change frequency and total water loss (Table 1). The subsequent increased total water loss beyond one standard deviation of CRHO-06 can be explained by the animal's urination in its chamber during the trial period. Examination of the comparison between observed and theoretically predicted V_{CO_2} (Table 2) suggests that observed values were credible and could be used in calculating total CO_2 production. The difference between mass loss and Drierite water loss (mass loss difference) corresponds with the production of water as the result of aerobic metabolism. Theoretically, it is then expected that the rest of the mass loss was the result of the animal metabolizing its energy stores. As the animal becomes increasingly dehydrated, there is an increased need to produce more water by metabolic needs, and this can be detected by a shift in RQ and/or an increased metabolic rate. The positive correlation between water loss and V_{CO_2} supports this, and it is expected that the isotopic data will confirm it once the source of the unrealistic isotope values is determined.

Although the gathered stable isotope data was not analyzed, application of the DLW method was expected to yield metabolic rates not significantly different from those determined using open-flow respirometry. The two-sample technique is typically the only approach available to field researchers interested in measuring field metabolic rate over relatively long periods of time in organisms where other types of calorimetric measurements would be difficult or impossible (Jones et al. 2009). The noninvasive and nonrestrictive nature of the DLW method as well as its ease of application makes it ideal for the measurement of total daily energy expenditure in free-living organisms. There are also various human applications to the method

including but not limited to studies measuring energy-expenditure, total body water, water intake, and metabolizable energy intake.

Validation of this technique provides researchers with increased confidence of experimentally determined metabolic rates using DLW, especially when open-flow respirometry is not an option for measurement, as well as improving our ability to detect responses in snakes to natural and artificial perturbations to their environment. However, we must acknowledge that in the field, where this method is often utilized, snakes are feeding based on their individual needs. Future validations of this technique should consider feeding the snakes, thereby incorporating a food energy input into the mass balance calculation, and therefore improving precision for the more practical uses of the method, such as quantifying the cost of specific activities like ecydysis and mating effort (Friesen et al 2015). Also, additional research on a broader array of taxa is needed to increase use amongst other taxa.

Literature Cited

Beaupre S.J. 2008. Annual variation in time-energy allocation by Timber Rattlesnakes (*Crotalus horridus)* in relation to food acquisition. In W.K. Hayes, K.R. Beaman, M.D. Cardwell, and S.P. Bush, eds. The Biology of Rattlesnakes. Loma Linda University Press, Loma Linda, California.

Beaupre S.J. and F. Zaidan III. 2001. Scaling of CO_2 production in the Timber Rattlesnake (*Crotalus horridus*), with comments on cost of growth in neonates and comparative patterns. Physiological and Biochemical Zoology 74:757-768.

Bush M. and J. Smeller. 1978. Blood collection & injection techniques in snakes. Veterinary Medicine/ Small Animal Clinician 73:211-214.

Butler P. J., J.A. Green, I.L. Boyd, and J.R. Speakman. 2004. Measuring metabolic rate in the field: the pros and cons of the doubly labeled water and heart rate methods. Funct Ecol 18:168-183.

Friesen C.R., D.R. Powers, P.E. Copenhaver, and R.T. Mason. 2015. Size dependence in non-sperm ejaculate production is reflected in daily energy expenditure and resting metabolic rate. Journal of Experimental Biology 218: 1410-1418.

Fry B. 2006. *Stable Isotope Ecology*. New York, NY: Springer Science.

Jones T. T., M.D. Hastings, B.L. Bostrom, R.D. Andrews, and D.R. Jones. 2009. Validation of the use of doubly labeled water for estimating metabolic rate in the green turtle (Chelonia mydas L.): a word of caution. Journal of Experimental Biology 212:2635-2644.

Lifson N. and R. McClintock. 1966. Theory and use of the turnover rates of body water for measuring energy and material balance. Journal of Theoretical Biology 12:46-74.

Lighton J.R.B. 2008. Measuring Metabolic Rates: A Manual for Scientists. Oxford University Press, New York.

Nagy K.A. 1980. CO_2 production in animals: analysis of potential errors in the doubly-labeled water method. American Journal of Physiology 238R:466-473.

Nagy K.A. 1983. The doubly-labeled water ($^3H_2$ ^{18}O) method: a guide to its use. University of California Los Angeles Publications No. 12-1417.

Nagy K.A. 1989. Doubly-labeled water studies of vertebrate physiological ecology. In P.W. Rundel, J.R. Ehleringer, and K.A. Nagy, eds. Stable Isotopes in Ecological Research. Springer-Verlag, New York.

Nagy K.A. 2005. Field metabolic rate and body size. Journal of Experimental Biology 208:1621-1625.

Nagy K.A. and D. P. Costa. 1980. Water flux in animals: analysis of potential errors in the tritiated water method. American Journal of Physiology 238R:454-465.

Nagy K.A., D.J. Morafka, and R.A. Yates. 1997. Young Desert Tortoise survival: energy, water, and food requirements in the field. Chelonian Conservation and Biology 2:396-404.

Porter W.P., J.C. Munger, W.E. Stewart, S. Budaraju, and J. Jaeger. 1994. Endotherm energetics: from a scalable individual-based model to ecological applications. Australian Journal of Zoology 42:125-162.

Speakman J.R. 1997. Doubly Labelled Water: Theory and Practice. Chapman & Hall, New York.

Speakman J. R. 2014. Should we abandon indirect calorimetry as a tool to diagnose energy expenditure? Not yet. Perhaps not ever. Commentary on Burnett and Grobe (2014). Molecular Metabolism 3(4):342–344.

Tolkamp B.J., G.C. Emmans, J. Yearsley, and I. Kyriazakis. 2002. Optimization of short-term animal behaviour and the currency of time. Animal Behaviour 64:945–995.

Figures and Tables

Figure 1. Open-flow respirometry setup for measuring metabolic rates (V_{CO_2} and V_{O_2}). Scrubbed air flowed from a purge gas generator through a regulator before entering a manifold and being diverted into each chamber. Each chamber was connected to a channel line that linked to its own corresponding Drierite column where ex-current air was scrubbed of water vapor before entering a multiplexer. Air leaving the multiplexer was pushed through an O_2 analyzer, which measured O_2 (ppm). Air then entered a mixing chamber along with scrubbed air from the Mass Flow Controller (MFC) to dilute the incoming gas stream before being subsampled. The gas stream was pulled though a second Drierite column and then a CO_2 analyzer that measured CO_2 (ppm). Each chamber was measured for 7.5 minutes every hour. Switching between chambers was dictated by a computer-controlled multiplexer. Along with each animal chamber, a baseline (line without a chamber or Drierite column) was measured at the top and bottom of each hour for 3.75 min each. The computer program, Expedata was used to control the multiplexer, record data, and process data for analysis. The program recorded the following every 5 seconds per chamber: subsampler A flow rate (mL/min), O_2 (ppm), subsampler B flow rate (mL/min), CO_2 (ppm), and the temperature of the chamber (˚C).

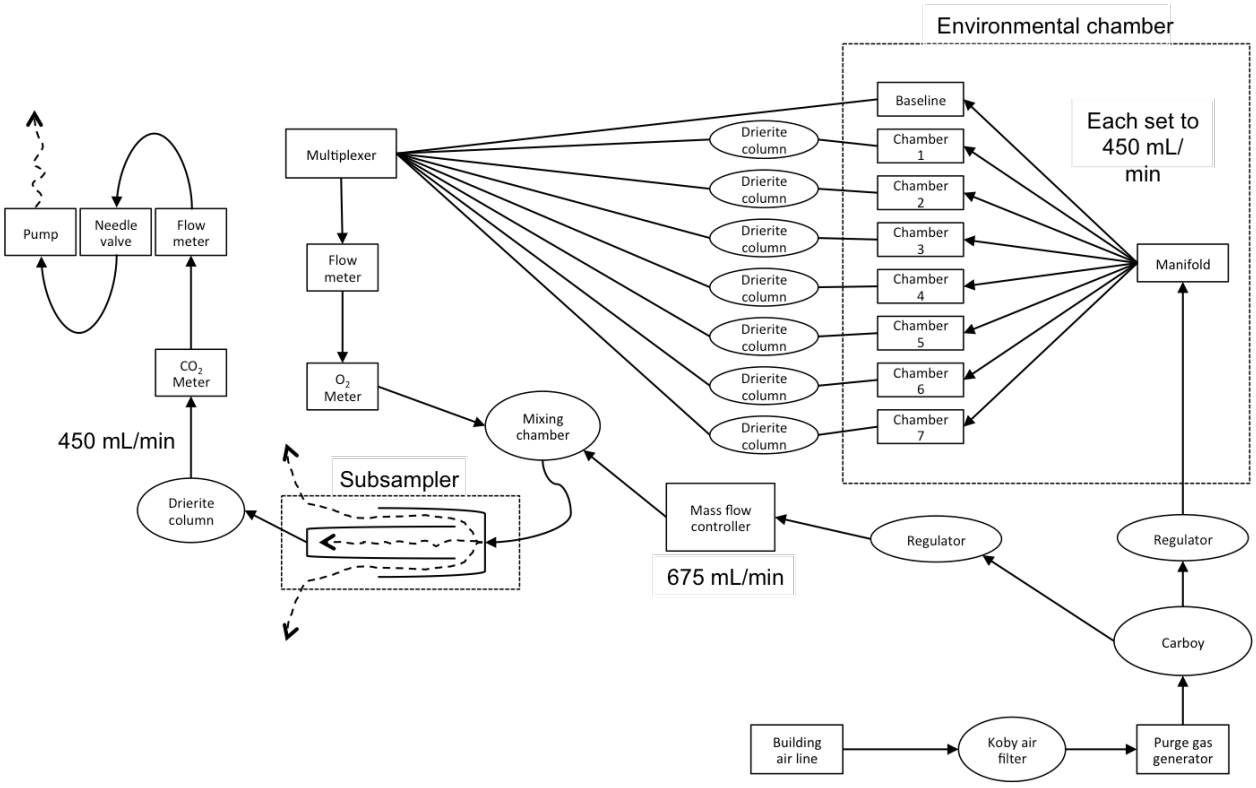

Table 1. The total measured water loss for each animal calculated as the summed pre- and post-run Drierite column mass difference for each snake, and the frequency Drierite columns were changed in accordance to their turnover rate.

ID	Total Water Loss (g)	Column Change Frequency
CRHO-01	23.4011	21
CRHO-02	14.5066	20
CRHO-03	23.1560	20
CRHO-04	45.0084	43
CRHO-05	39.4032	41
CRHO-06	62.1339	45
CRHO-07	40.7768	44
Average	35.4837	33.4
SD	16.2395	12.3

Table 2. Observed average V_{CO_2} compared to theoretically calculated V_{CO_2} values per animal and averaged over all animals.

ID	Mass (g)	Average V_{CO_2} (mL/h)	CI 95%	Predicted V_{CO_2} (mL/h)	Difference (Observed - Predicted)
CRHO-01	357.26	2.851	0.0710	3.837	-0.985
CRHO-02	147.45	1.589	0.0511	1.929	-0.340
CRHO-03	241.50	2.972	0.0776	2.830	0.142
CRHO-04	650.53	5.494	0.2981	6.112	-0.618
CRHO-05	549.37	6.063	0.2876	5.360	0.703
CRHO-06	484.26	3.587	0.1674	4.859	-1.272
CRHO-07	616.72	5.392	0.1930	5.864	-0.472
Average	435.30	3.993		4.399	-0.406
SD	191.99	1.672		1.586	0.667

Note. Predicted V_{CO_2} was determined using the following equation: $\dot{V}_{CO_2} = 0.00124 W^{.777} 10^{.0590T}$

W = weight (g); T = temperature of chamber (^{o}C) The environmental chamber temperature was 25.54^{o}C for the entirety of the trial.

Table 3. Measured initial and final mass, mass loss, injectate dosage, Drierite determined water loss, mass loss difference, and total CO_2 production for 7 snakes in DLW validation.

ID	Initial mass (g)	Final mass (g)	Mass loss (g)	Injected DLW dose (g)	Drierite water loss (g)	Mass loss difference (g)	Total CO_2 production (mL)	Calculated water loss (g)	CO_2 DLW
CRHO-01	357.26	332.45	24.81	0.1635	23.40	1.41	1328.35		
CRHO-02	147.45	131.20	16.25	0.0564	14.51	1.74	739.73		
CRHO-03	241.50	218.02	23.48	0.1168	23.16	0.32	1384.29		
CRHO-04	650.53	603.99	46.54	0.2746	45.01	1.53	2559.47		
CRHO-05	549.37	508.94	40.43	0.2300	39.40	1.03	2825.22		
CRHO-06	484.26	413.94	70.32	0.2105	62.13	8.19	1670.69		
CRHO-07	616.72	573.16	43.56	0.2720	40.78	2.78	2512.48		
Average	435.30	397.39	37.91	0.1891	35.48	2.43	1860.03		
SD	191.99	179.60	18.31	0.0815	16.24	2.65	779.58		

Table 4. Background isotope levels (BKG), post-injectate equilibration isotope levels (1S), and final isotope levels (2S) for seven Timber Rattlesnakes used in DLW validation. Water background isotope levels and post-injectate isotope levels for three samples of known water mass used in dilution validation.

ID	Delta ^{2}H	Delta ^{18}O
CRHO-01_BKG	-4.97	1.66
CRHO-01_1S	959.08	165.91
CRHO-01_2S	889.04	154.77
CRHO-02_BKG	-13.75	1.57
CRHO-02_1S	804.73	135.42
CRHO-02_2S	742.15	122.54
CRHO-03_BKG	-9.26	1.42
CRHO-03_1S	947.04	164.77
CRHO-03_2S	945.30	166.51
CRHO-04_BKG	-9.12	1.64
CRHO-04_1S	847.89	143.77
CRHO-04_2S	842.73	147.00
CRHO-05_BKG	-9.04	3.89
CRHO-05_1S	929.30	180.91
CRHO-05_2S	836.63	135.84
CRHO-06_BKG	-8.57	6.67
CRHO-06_1S	832.75	152.02
CRHO-06_2S	831.59	140.35
CRHO-07_BKG	-10.76	4.80
CRHO-07_1S	898.14	148.56
CRHO-07_2S	744.45	114.22
Water_BKG	-41.22	-5.29
DD_1	800.10	134.89
DD_2	869.97	141.61
DD_3	946.08	160.75